

Publication Title:

HEPATOCELLULAR CARCINOMA ONCOGENE

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07H 15/12, C12Q 1/68 C12N 15/00	A1	(11) International Publication Number: WO 91/09045 (43) International Publication Date: 27 June 1991 (27.06.91)
(21) International Application Number: PCT/US90/07333 (22) International Filing Date: 13 December 1990 (13.12.90) (30) Priority data: 451,953 19 December 1989 (19.12.89) US 575,524 31 August 1990 (31.08.90) US (71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, UNITED STATES DEPARTMENT OF COMMERCE [US/US]; Washington, DC 20231 (US). (72) Inventor: YANG, Stringner, Sue ; 6625 Marywood Road, Bethesda, MD 20817 (US). (74) Agents: OLIFF, James, A. et al.; Oliff & Berridge, P.O. Box 19928, Alexandria, VA 22320 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: HEPATOCELLULAR CARCINOMA ONCOGENE (57) Abstract The present invention relates to an oncoprotein specific for hepatocellular carcinomas and to a nucleotide sequence that codes for such a protein. The invention further relates to screening and diagnostic methodologies (and kits based thereon) that make use of the oncoprotein (or antibodies specific for same) and the nucleotide sequence.		

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HEPATOCELLULAR CARCINOMA ONCOGENE

TECHNICAL FIELD

The present invention relates, in general, to a protein of hepatoma cells, and, in particular, to an oncoprotein that is an amplified gene expression product of hepatoma cells. The invention further relates to a nucleotide fragment coding for the oncoprotein, to a recombinant molecule that includes such a fragment and to cells transformed therewith. The invention further relates to methods of detecting the presence of hepatocellular carcinomas in a patient and to kits based thereon.

BACKGROUND INFORMATION

Epidemiological evidence has led to a strong etiological implication of several DNA viruses with the occurrence of certain cancers and other disorders in humans. These include the papillomavirus in cervical carcinoma (HPV 16) and in epidermodysplasia verruciformis (HPV 3 and 8); the Epstein-Barr virus in Burkitt's lymphoma; and the hepatitis B virus (HBV) in human hepatocellular carcinoma (Beasley et al, In: Vyas GN, Dienstag JL, Hoofnagle JH, eds. Viral hepatitis and liver disease. Orlando, FL, Grune and Stratton, 1984, 209-224). These observations, together with the correlation of retroviral infection such as HTLV-I in Adult T-cell leukemia asserts the possible role of infectious viruses acting as transducing agents in the pathogenesis of these aforementioned human neoplasms and disorders.

The mechanism(s) by which infectious viruses exert their oncogenicity is believed to be mediated by DNA recombination with the host cell DNA. The mammalian genome contains certain genes, designated proto-oncogenes, that can acquire oncogenic properties upon transduction into the genome of acute transforming retroviruses (Bishop, Ann. Rev. Biochem. 1983, 52:301; Bishop, Cell 1985, 42:23). In certain human cancers (e.g. T24 and EJ human bladder carcinoma) it has been well documented that the identified transforming gene (H-ras-1 locus) relates to the v-rasH of the Harvey murine sarcoma virus. Among the

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proto-oncogenes and oncogenes, the ras family has been thoroughly characterized and studied with respect to activation and expression in human neoplasms. When a proto-oncogene undergoes point-mutation (e.g. c-rasH) or
5 rearrangement (e.g. n-myc), such changes can lead to a loss of cell regulation in differentiation and growth, and eventually oncogenesis.

Recently, a transforming DNA sequence from a human (Mahlavu) hepatocellular carcinoma, hhc^M, has been identified and molecularly cloned as part of a large fragment
10 (Yang et al, J. Gen. Virol. 1982, 63:25; Yang et al, Environmental Health Perspectives 1985, 62:231). A number of hhc^M related DNA clones from several other human hepatocellular carcinomas have been isolated that exhibited
15 nil to moderate cell transforming activity on NIH/3T3 cells. Two have been partially characterized and they are a moderately cell-transforming gene from Mahlavu hepatocellular carcinoma (hhc^M) and a putative cellular homologue (c-hhc) isolated from normal human liver DNA, which
20 has no cell-transforming activity. The biological activities of two molecular clones of hhc^M and a Korean hhc^K and c-hhc have been characterized and compared (Yang et al, Leukemia 1988, 2(12 Supplement):102S). Amplification of the hhc^M sequence in the various genomic DNAs of hepatomas
25 from 2 Chinese, one African and 17 Korean sources, was observed and compared with the distribution of integrated HBV DNA sequences in the same hepatomas in order to provide some insight into the possible role of hhc^M.

The present invention relates to an oncoprotein specific for hepatocellular carcinomas and to a nucleotide
30 sequence that codes for such a protein. The invention further relates to diagnostic and screening methodologies (and kits based thereon) that make use of the oncoprotein (or antibodies specific for same) and the nucleotide
35 sequence.

SUMMARY OF THE INVENTION

It is one object of the invention to provide a

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hepatocellular oncoprotein and a nucleotide sequence coding for same.

It is another object of the invention to provide a diagnostic test for the presence of hepatocellular carcinomas as well as preneoplastic or pathological conditions of the liver.

Further objects and advantages of the present invention will be clear to one skilled in the art from the description that follows.

In one embodiment, the present invention relates to a DNA fragment coding for the amino acid sequence set forth in Figure 1 or an allelic variation of that sequence, or a unique portion thereof.

In another embodiment, the present invention relates to a recombinant DNA molecule comprising:

- i) a vector, and
- ii) the above-described DNA fragment.

In a further embodiment, the present invention relates to a host cell transformed with the above-described recombinant DNA molecule.

In another embodiment, the present invention relates to a nucleotide fragment sufficiently complementary to the above-described DNA fragment to hybridize therewith.

In a further embodiment, the present invention relates to a protein having the amino acid sequence set forth in Figure 1 or an allelic variation of that sequence, or a unique portion thereof.

In another embodiment, the present invention relates to antibodies (polyclonal and/or monoclonal) specific for the above-described protein.

In a further embodiment, the present invention relates to a process of producing the above-described protein comprising culturing a host cell transformed with the above-described recombinant DNA molecule under conditions such that the DNA fragment is expressed and the protein thereby produced; and isolating the protein.

In another embodiment, the present invention

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relates to a method of detecting the presence of the above-described protein in a sample comprising:

5 i) contacting the sample with an antibody specific for the protein under conditions such that binding of the antibody to the protein can occur, whereby a complex is formed; and

ii) assaying for the presence of the complex.

10 In another embodiment, the present invention relates to a method of detecting the presence of a nucleotide sequence coding for the above-described protein in a sample comprising: i) contacting the sample with a nucleotide fragment sufficiently complementary to the nucleotide sequence to hybridize therewith under conditions such that hybridization can occur, whereby a complex is formed, and

15 ii) assaying for the presence of the complex.

In a further embodiment, the present invention relates to a method of diagnosing the presence of hepatocellular carcinoma in a patient comprising:

20 i) contacting a biological sample from the patient with the above-described antibody under conditions such that binding of the antibody to the protein present in the sample can occur, whereby a complex is formed; and

ii) assaying for the presence of the complex.

25 In another embodiment, the present invention relates to a method of diagnosing the presence of hepatocellular carcinoma in a patient comprising:

30 i) contacting nucleic acid sequences derived from a cellular sample from the patient with the above-described nucleotide fragment under conditions such that hybridization can occur, whereby a complex is formed; and

ii) assaying for the presence of the complex.

35 In another embodiment, the present invention relates to a diagnostic kit for detecting the presence of the above-described protein in a sample comprising a container means having disposed therein antibodies specific for the protein.

In a further embodiment, the present invention relates to a diagnostic kit for detecting the presence of

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a nucleic acid sequence coding for a protein having the amino acid sequence set forth in Figure 1 or an allelic variation of the sequence, or a unique portion thereof, comprising a container means having disposed therewithin the above-described nucleotide fragment.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the complete nucleotide sequence of hhc^M , and the amino acid sequence of a 52,000 dalton protein encoded within its open reading frame.

Figures 2 shows the construction of hhc^M -LacZ chimeric plasmid for the production of the hhc^M 52 kD protein.

Figure 3 shows the Aflatoxin B₁ epoxide binding on high molecular weight DNAs prepared from human hepatocellular carcinoma (Mahlavu), human normal liver and from murine (NIH/3T3) fibroblasts.

Figure 4 shows the identification of the dG bound by AFB₁ epoxide within the hhc^M (PM-1) DNA by a modified Maxam-Gilbert sequencing method. Nucleotide sequences are specified on the side. The left panel illustrates ladder for all four 6 deoxynucleotides and AFB₁-dG; only native dG and AFB₁-dG were given in all other three panels on the right. aG = AFB₁ bound dG at all time; °G = dG that was not reacted with AFB₁; whereas °G = moderately preferred dG.

Figure 5 shows the kinetic analysis of protein production in E. coli cells harboring pJZ102. Plasmid pJZ102 and control plasmid pJZ101 were cultured in E. coli cells until cell density reached a Klett reading of 80, at which point the inducer, IPTG (final concentration, 10^{-3} mol), was added to activate transcription from the lac promoter for the production of the chimeric hhc^M -lac 52-kD protein. One ml samples of the cultures were removed at specified times, pelleted by centrifugation and lysed, and the proteins were denatured by boiling in Laemmli buffer. Equivalent aliquots of each sample were applied and analyzed by SDS-polyacrylamide gel electrophoresis as

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described in (Somerville et al., Structural and Organizational Aspects of Metabolic Regulation: UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 133, p. 181-197. New York: Alan R. Liss, Inc. 1990). The lanes represent: (a) pJZ102 + ITPG at time zero; (b) pJZ102 - ITPG at time zero, and 20 hours (c); pJZ102 + ITPG at 30 minutes (d), 4 hours (e), 7 hours (f), and 20 hours (g). Dark field microscopy of pJZ102 transformed E. coli cells + ITPG at 0 time (a'), 30 minutes (b'), 4 hours (e'), 7 hours (f'), and 20 hours (g'). Prestained molecular weight markers (m) in kD are 130 (faint band on top), 94, 75, 50, 39, 27, 17.

Figures 6A and 6B show purified hhc^M fusion protein p52 produced in bacteria (Figure 6A) and specificity of a polyclonal anti-p52 IgG (Figure 6B). Figure 6A shows the SDS-polyacrylamide gel electrophoresis of bacterially expressed p52. All conditions for the bacterial expression of chimeric hhc^M-lac fusion proteins were as described in Figure 5. Lanes d, e, and e' represent total cell extracts of pJZ102-bearing E. coli cells (in varying amounts) induced by IPTG and lane f represents the total cell extracts of a negative control pJZ101-bearing E. coli cells. Lanes a (5 μ l), b (15 μ l) and c (1 μ l) depict different amounts of gel purified p52 that was used to immunize rabbits. Lane m depicts pre-stained molecular markers in kD of 75, 57, 50, 39, 27, 17.

Figure 6B shows the reactivity of a polyclonal anti-p52. Anti-p52 polyclonal IgG was raised by immunizing rabbits. SDS polyacrylamide gel purified p52 at 0.8 to 1.0 mg each was used to immunize the New Zealand White rabbit by standard techniques. Two booster injections were given. Detergent (0.2% SDS) lyzed samples corresponding to 0.2 ml of packed human hepatoma cells (1/3:v/v) including Mahlavu hepatocellular carcinoma, Hp3p21.7 and HPG2, and pB^rpM-1 transfected BRL-1 tumor cells and control BRL-1 cells and p52, at 10 μ l each were applied to sample well and allowed to diffuse and cross-react overnight against the polyclonal anti-p52 IgG.

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Results were recorded at 48 hours.

Figure 7 shows the DNA-DNA hybridization against ^{32}P -hhc^H DNA.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention relates to an oncoprotein coded for by a transforming nucleotide sequence of hepatocellular carcinomas and to the transforming sequence itself. The invention further relates to unique portions (i.e., at least 5 amino acids) of the oncoprotein, and to
10 nucleotide sequences (fragments) that code for such polypeptides. The invention further relates to nucleotide segments sufficiently complementary to the above-described nucleotide sequences (fragments) to be used as probes for detecting the presence of such nucleotide sequences
15 (fragments). The invention also relates to diagnostic and screening methodologies for use in detecting the presence of hepatocellular carcinomas (as well as preneoplastic or pathological conditions of the liver) in a warm blood animal.

20 The oncoprotein of the present invention is an amplified gene expression product of hepatoma cells that is specifically related to hepatomas. The protein can have the complete sequence given in Figure 1, in which case it is designated hhc^H. The protein can also have the
25 amino acid sequence of a molecule having substantially the same properties (e.g., immunological) as the molecule given in Figure 1 (for example, allelic forms of the Figure 1 sequence). Alternatively, the protein (or polypeptide) of the invention can have an amino acid
30 sequence corresponding to a unique portion of the sequence given in Figure 1 (or allelic form thereof).

 The protein can be present in a substantially pure form, that is, in a form substantially free of proteins and nucleic acids with which it is normally associated in
35 the liver. The oncoprotein of the invention, including that made in cell-free extracts using corresponding mRNA, and the oncoprotein made using recombinant techniques, can be purified using protocols known in the art. The onco-

protein, or unique portion thereof, can be used as an antigen, in protocols known in the art, to produce antibodies thereto, both monoclonal and polyclonal.

5 In another embodiment, the present invention relates, as indicated above, to nucleotide sequences (fragments) (including cDNA sequences) that encode the entire amino acid sequence given in Figure 1 (the specific DNA sequence given in Figure 1 being only one example), or
10 any unique portion thereof. Nucleotide sequences to which the invention relates also include those coding for proteins (or polypeptides) having substantially the same properties (e.g., immunological) of the hhc^M polypeptide (for example, allelic forms of the amino acid sequence of Figure 1). The invention further relates to nucleotide
15 segments sufficiently complementary to the above-described nucleotide sequences (fragments) to hybridize therewith (e.g. under stringent conditions).

In another embodiment, the present invention relates to a recombinant molecule that includes a vector
20 and a nucleotide sequence (fragment) as described above (advantageously, a DNA sequence coding for the molecule shown in Figure 1 or a molecule having the properties thereof). The vector can take the form of a virus or a plasmid vector. The sequence can be present in the vector
25 operably linked to regulatory elements, including, for example, a promoter (e.g., the LacZ promoter). The recombinant molecule can be suitable for transforming procaryotic or eucaryotic cells, advantageously, protease deficient E. coli cells.

30 A specific example of a recombinant molecule of the invention is shown in Figure 2. In this example, the hcc^M nucleotide sequence is placed in a chimeric construct by replacing the codons of the original N-terminus 18 amino acids of the hhc^M p52kD with the procaryote LacZ
35 expression/translation sequence plus codons for 11 amino acids by appropriate recombinant DNA manipulations (Yang et al. Proc. of the XIV Inter. Symp. Sponsored by the International Association for Comparative Research on

Leukemia and Related Diseases Nov. 1989 (Vale, Colorado)). Driven by the LacZ promoter, the resultant chimeric gene is expressed at high levels in a protease deficient E. coli mutant at 30°C. In a further embodiment, the present invention relates to a host cell transformed with the above-described recombinant molecule. The host can be procaryotic (for example, bacterial (advantageously E. coli)), lower eucaryotic (i.e., fungal, including yeast) or higher eucaryotic (i.e. mammalian, including human). Transformation can be effected using methods known in the art. The transformed host cells can be used as a source for the nucleotide sequence described above (which sequence constitutes part of the recombinant molecule). When the recombinant molecule takes the form of an expression system (see specific construct described above), the transformed cells can be used as a source for the oncoprotein.

The oncoprotein and nucleic acid sequence of the present invention can be used both in a research setting (for example, to facilitate an understanding of how and why hepatocellular carcinomas develop) and in a clinical setting to, for example, diagnosis (and/or screening) the presence and/or progress of hepatocellular carcinomas (as well as preneoplastic or pathological condition of the liver).

The diagnostic/screening methodologies referred to above can be carried out using antisera or monoclonal antibodies (produced using known techniques) against the oncoprotein (or unique portions thereof) of the invention. For example, the diagnostic method can take the form of an immunoassay that can be used with urine or serum samples of patients at high risk for hepatocellular carcinoma (e.g. chronic hepatitis carriers) and/or of populations in the geographically identified hot-spots of liver cancer (e.g. Chitung Province of China). The screening immunoassay can be of the simple dip-stick type where binding of one member of the antigen/antibody pair, attached to the stick, with the other member of the pair, present in the

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sample, is accompanied by a color change (such dip-stick type assays have been described for use with a variety of binding pairs). Such simple tests would be easily and widely applicable to populations in areas where analytical electrophoresis equipment (required for detecting alpha-fetoprotein levels in patients' sera, which levels are currently used in screening and diagnosing the presence of hepatocellular carcinomas) may not be readily available.

The diagnostic methods of the invention can also take the form of a histochemical diagnostic tests involving the use of antibodies against the protein or polypeptide of the invention. Such a test can be used on frozen or prefixed liver thin section samples to enable a more definite diagnosis of liver cancer.

The diagnostic methods of the invention can also involve the use of nucleic acid probes sufficiently complementary to a portion of the nucleic acid sequence of the invention to hybridize thereto. Such probes can be used to detect the presence of the endogenous sequence, for example, following electrophoresis of genomic DNA digested with appropriate restriction enzymes. The probe can be labelled, for example, with ^{32}P , to facilitate detection.

The invention further relates to diagnostic/screening kits for use in carrying out the above methods. The kits can comprise, for example, the above-described antibodies specific for the oncoprotein (or polypeptide) of the invention or, alternatively, the above-described nucleic acid probes, together with any ancillary reagents (e.g., buffers, detectable markers, enzyme substrates, etc.) necessary to conducting the test.

The invention is described in further detail in the following non-limiting Examples.

Examples

The following protocols are referenced in the Examples that follow:

Molecular cloning of hhc^M

Genomic DNA purified from human normal liver and Mahlavu (African) hepatocellular carcinoma (HHC), as described below, were subjected to complete digestion by HindIII restriction endonuclease. (Other restriction endonucleases including BamHI, EcoRI and PstI, were also used for isolating genomic DNA fragments from HHC and liver DNA in an attempt to clone HHC DNA sequences; the clones isolated from these efforts were not successful with respect to transfection studies.) The DNA samples both [³H]aflatoxin B₁ (AFB₁)-epoxide bound (as described below) and unbound, were separated into 180 fractions by polyacrylamide gel electrophoresis. Specificity of [³H]AFB₁-epoxide per µg of DNA was determined. Fractions with significant [³H]AFB₁-epoxide specific activity were used in DNA transfection assay on NIH3T3 cells as described below. Fractions showing positive focus formation indicating positive cell transformation, were identified and the parallel unbound DNA fractions were molecularly cloned by ligation onto the HindIII site of pBR322, pBR325 and/or Puc 8 plasmid DNAs for transformation of E. coli HB101 cells as described elsewhere (Yang et al., J. Gen. Virol. 1982, 63:25). Primary selection of the resultant clones was thus based on (1) the sensitivity to tetracycline, and/or color change associated with the disruption of the lacZ operon containing the B-galactosidase coding sequence of the plasmid; and (2) the capability of cell-transformation in transfection assays on NIH3T3 cells with or without AFB₁ binding; (3) the presence of human sequence in colony-hybridization and DNA-DNA hybridization against [³²P]probes prepared from human Alu sequence (Lawn et al., Cell 1978, 15:1157) and also [³²P] labelled HindIII digested MAH HHC DNA fragments; and (4) [³H] AFB₁-epoxide binding on the DNA fragments. After screening over 30,000 clones by these quadruple technical approaches including [³H]AFB₁ binding, transfection assay on NIH3T3 cells and DNA-DNA hybridization against the [³²P]Alu and [³²P]HindIII

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MAH HHC DNA probes, three clones were isolated. One particular 3.1 kb DNA restriction fragment constitutes the hhc^M DNA.

Preparation of plasmid DNA and AFB₁ binding

5 The clone used in these studies has been referred to as PM-1. Plasmid DNA was prepared by the Holmes' method, i.e. the rapid heating method, followed by CsCl₂-ethidium bromide isopycnic centrifugation at 180,000xg for 20 hrs (Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1982). The
10 banded PM-1 DNA was then purified free of ethidium bromide by isopropanol extraction and exhaustive dialysis against TEN buffer. A yield of 25 to 50 µg of total plasmid DNA per 5 ml of culture was generally obtained. The 3.1 kb
15 hhc^M DNA was then separated from PUC 8 DNA and other contaminants by digesting the PM-1 DNA with HindIII endonuclease and then subjecting to agarose gel electrophoresis and electroelution of the separated 3.1 kb band. The resultant 3.1 kb hhc^M DNA was homogeneously purified
20 and used in AFB₁ activation experiments.

 The hhc^M 3.1 kb DNA was also cloned into a pSVneo vector that carried a murine retroviral (Moloney) LTR, SV40 promoter and part of the T antigen besides the neomycin resistance gene. This clone, rpMpN-1, is ex-
25 pressed at a significantly higher level when transfected into cells and offers special advantages for transfection assay.

 [³H]AFB₁ at 15 Ci/mmol specificity was acquired from Morales Laboratory, CA. It was further purified by
30 HPLC to homogeneity and the resultant single peak of [³H]AFB₁ had the specific activity of 9,250 cpm/pmol. It was used in activation reactions with either mixed function oxidases freshly prepared from liver microsomal preparation or by the chemical peroxidation reaction using
35 perchlorobenzoic acid and methylene chloride as described earlier (Bennett et al., Cancer Res. 1981, 41:650; Garner et al., Chem. Biol. Interact. 1979, 26:57). Binding of

[³H]AFB₁ epoxide with either high molecular weight HHC or plasmid DNA was monitored by kinetic analysis (Yang et al. Environmental Health Perspective 1985, 62:231 and Modali and Yang, Monitoring of Occupational Genotoxigants pp. 147-158 (1986)). Samples withdrawn at each time point was washed free of unbound [³H]AFB₁ epoxide with chloroform, and ethanol precipitated prior to redissolving the [³H]AFB₁-DNA in Tris-EDTA-NaCl (TEN) buffer for transfection assay or sequence analysis.

10 Cells, tissue culture and transfection assay

NIH/3T3 cells, passage 6 to 11, and Buffalo rat liver cells (BRL-1) for transfection assays, were maintained in Dulbecco's modified Eagle's media supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 units ml⁻¹) and streptomycin (25 µg ml⁻¹) (DMEM) in a 5% CO₂ atmosphere, at 37°C.

DNA transfection was carried out as described earlier (see Yang et al. 1985 and Modali and Yang 1986, referenced above). Optimal conditions were achieved by carefully titrating the pH curve for the DNA-calcium phosphate complex mixture; it was usually found that pH 6.75 ensured a fine complex precipitation.

20 Preparations of DNA and RNA from tissue culture cells and tumor tissues

25 Total high molecular weight (HMW) DNA was extracted and purified from tissue culture cells and tumor tissues as described elsewhere (Yang et al., 1985 referenced above). The HMW DNA thus purified, has been subjected to proteinase K digestion, first sequential chemical purification with phenol-cresol, chloroform-isoamyl alcohol, ether and ethanol-NaCl precipitation, followed by RNase digestion and a second sequential chemical purification. The purified DNAs were then dialyzed against TEN buffer for use in experiments. Total RNA was extracted from tissue culture cells and prepared as described previously (Maniatis et al., 1982 referenced above). Poly A rich RNA was obtained by affinity separation with oligo dT cellulose (Collaborative Research, MA.) column elution.

Tumorigenesis

Transformed cells, cloned out from the transfected cell culture by either cloning cylinder method or terminal dilution method, were expanded and inoculated at 10^4 to 10^6 cells into athymic Swiss nu/nu mice subcutaneously. Tumorigenesis in the challenged mice was monitored closely.

Nucleotide sequence analysis and site-targeted mutagenesis

Nucleotide sequencing of the hhc^M 3.1 kb and variants produced by site-targeted mutagenesis were carried out by the standard Maxam-Gilbert Methods in Enzymology 1980, 65:499 and the Sanger (M13) dideoxy sequencing methods (Maniatis et al., 1982 referenced above).

Specified oligonucleotide sequence of 20 mers carrying the targeted dG--->T mutation were synthesized by the Applied Biosystem oligonucleotide synthesizers. They were used as templates in generating the mutated clones. Mutant DNA clones were produced in accordance with the protocol provided by and using the oligonucleotide-directed in vitro system of Amersham (Arlington Hts., IL). DNAs of the mutated clones were verified by nucleotide sequencing. Effects of these site-targeted mutagenized DNA were analyzed by potentiation of cell-transformation in transfection assay on NIH/3T3 cells and RNA expressions in transfected cells using the BRL dot-blot technique (Bethesda Research Laboratory, Rockville, MD).

Example I

Dosimetry of AFB₁ binding and potentiation of hhc^M cell-transformation capability on NIH/3T3 cells

AFB₁ epoxide binds high molecular weight DNAs prepared from human hepatoma, human liver and mouse NIH/3T3 cells efficiently (Fig. 3). The initial rates in each binding kinetic were extremely rapid. The rates of AFB₁-epoxide binding to human normal liver or hepatoma DNA and to murine NIH/3T3 cell DNA became significantly different after one minute of binding reaction. The MAH HHC DNA showed a greater rate of binding than normal liver

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DNA and all the dG targets became saturated earlier, whereas AFB₁ epoxide bound the normal liver DNA at a slower rate but eventually saturated all the dG targets at a slightly lower level. The human DNAs showed a higher level of AFB₁ binding than the murine NIH/3T3 cell DNA. The overall AFB₁ specific activity, i.e. AFB₁-dG adduct, was found to be about one dG bound per 10 nucleotides among these high molecular weight double stranded DNAs. This overall specificity also took into consideration the existence of secondary or tertiary structure of the high molecular weight DNAs. AFB₁ epoxide binding on linearized 3.1 kb double stranded hhc^M DNA was consistently found to be 4 to 8 dG bound per 10⁴ nucleotides. This higher binding capability reflects the relatively easy accessibility of dG within the linearized double stranded PM-1 DNA by AFB₁ epoxide and should not be compared with the efficiency of AFB₁-dG adduct formation with high molecular weight native double-stranded DNA.

Within a finite dosimetry the binding of AFB₁ epoxide with dG potentiates the cell-transformation capability of hhc^M by 10 to 20 fold as seen in the experiment illustrated in Table 1.

Table 1. AFB₁ Dose-dependent Activation of PM-1 DNA in Transformation of NUB/3T3 Cells

	DNA Source	AFB ₁ femtomole per 100 ng DNA	Number of Foci per 100 ng DNA
5	hhc ^M (PM-1)	0	15 x 10 ⁻¹
	C-Ha-ras-1	0	465
	c-K-ras-1	0	0
10	c-hhc (human liver homolog)	0	0
	<u>E. coli</u>	0	0
	hhc ^M (PM-1)	0	15 x 10 ⁻¹
	hhc ^M (PM-1)	5	18
15		14	26
		24	66
		35	3
	c-hhc	0	0
		8	0
20		15	0
		30	0
		40	0

25 AFB₁ binding and transfection assay were as described in Methods. Data were calculated on the basis of per 100 ng. In the assay with unbound hhc^M DNA the transfection assays were carried out with 500 ng to 1.5 ug of DNA in order to obtain reasonable foci formation on NIH/3T3 cells. Transfection with AFB₁-epoxide bound DNA was carried out at a range of 50 to 500 ng DNA. Data were normalized to show potentiation of hhc^M cell-transformation capability by AFB₁-epoxide activation.

35 Whereas the efficiency of unbound PM-1 DNA in transforming NIH/3T3 cells was usually observed at about 15 FFU/μg DNA the efficiency of AFB₁ epoxide activated PM-1 DNA was optimized at 66 FFU/100 ng DNA, an increase of 20 fold. The possibility of non-specific mutagenization accounting for this potentiation were considered. That this potentiation effect was due to free AFB₁ that diffused into the cell or recycling of AFB₁ adducts has been ruled out earlier with the appropriate control experiments which showed that activation of normal liver or E. coli DNA at the same dosimetry failed to activate any cell-transforming capability (Yang et al., 1985 referenced above).

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Moreover in this experiment with AFB₁ activated DNA from c-ras^k-1 or c-hhc, a normal human liver homolog to hhc^M as the appropriate controls, no cell-transformation of NIH/3T3 cells was obtained suggesting that AFB₁ epoxide activated PM-1 DNA was not a random phenomenon. Moreover the AFB₁ dose dependency of PM-1 DNA in cell-transformation efficiency (Table 1) further substantiated the specificity of AFB₁ epoxide binding in conferring the potentiation of cell-transformation. Whereas optimal dosimetry was seen at 24 femtomole AFB₁/100 ng of PM-1 DNA, at dosimetry beyond 45 femtomole per 100 ng of PM-1 DNA, an overkill effect was observed. No transformed foci were obtained in NIH/3T3 cells transfected with AFB₁ epoxide bound PM-1 DNA although human DNA was incorporated into the NIH/3T3 cells in a degraded form (Yang et al., 1985 and Modali and Yang, 1986 referenced above). This observation suggested that over activation of PM-1 DNA not only generated scissions in the molecule but possibly degradation leading to a loss of biological activity. It was also evident from these results that no more than one or at most a few AFB₁-dG adducts per PM-1 DNA molecule could be tolerated by the hhc^M DNA before the biological activity of the hhc^M DNA became compromised and at the risk of survival. Moreover the potentiation of hhc^M DNA in cell-transformation probably necessitates no more than one or at most a finite number of AFB₁ bindings.

EXAMPLE II

Specificity of the AFB₁-epoxide binding on dG's of PM-1 DNA

Deoxyguanine nucleotide of native DNA, when bound by AFB₁ epoxide, became alkali and therefore could be identified by piperidine cleavage; whereas unbound deoxyguanine nucleotide within the same native DNA would not cleave without dimethyl sulfide (alkali) treatment. Figure 4 shows the dG targets within the PM-1 DNA when bound at a saturation conditions. When the targeted sequences are evaluated in sets of tetranucleotides, an empirical formulation can be derived on the basis of the

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binding pattern of AFB₁ epoxide with the dG's in PM-1 DNA. Table 2 summarizes the nucleotide sequence in a set of tetranucleotides that were seen and targeted by AFB₁ epoxide. As shown in Figure 4, the dG within a sequence of any one of the following tetranucleotides of AGAG, AGTT, TGTT, TGAT, or AGAA, escaped attack by AFB₁ epoxide and hence showed no cleavage in the sequence without prior DMS treatment. This is confirmed by the distinct cleavage of dG as a result of AFB₁ epoxide attack on dG in a sequences of GGGC, CGGC, AGGC, TGGC or CGCG. Upon evaluating the various sequences in which a dG target could be accessed by AFB₁ epoxide, it can be concluded that within a double stranded DNA, the least likely dG target would be that flanked by dA and dT, i.e. category III. The most likely dG target would be that flanked by dG and/or dC, i.e. category I, and that tetranucleotide sequences in which dG is either preceded by dA or T and followed by dG and dC would be the moderately preferred targets of AFB₁ epoxide, i.e. category II. This, of course, does not take into consideration the secondary or the tertiary structure of the DNA in its natural state since these analyses were done on linearized double-stranded DNA. It should also be mentioned that whereas the dG binding affinity of AFB₁-epoxide was greatly affected by the vicinal nucleotides in the double-stranded PM-1 DNA, no specificity was observed with respect to AFB₁-epoxide binding to dG in single stranded DNA. The observations of Modali and Yang (1986 referenced above) were basically in agreement with others working on AFB₁ binding on OX174 and pBR 322 DNAs (Misra et al., Biochemistry, 1983, 22:3351).

Within the past two years, the nucleotide sequence of hhc^M has been resolved by a combination of Maxam-Gilbert nucleotide sequencing technique and the M13 dideoxy method using the BRL kilobase sequencing system. Applying these empirical rules in computer analysis of the hhc^M 3.1 kb nucleotide sequence, the most and moderately preferred dG targets within the various loci of hhc^M have

- 19 -

been predicted (Table 3). Although a maximum number of 60 dG targets was predicted on the basis of AFB₁-epoxide binding studies with linearized 3.1 kb hhc^M DNA, it was evident upon examining the possible secondary and tertiary structure of hhc^M sequence, that a much lower number of dG targets would be accessible by AFB₁-epoxide. Moreover, only a few such induced mutations would produce any effect of survival value.

- 20 -

Table 2. Vicinal Nucleotide Sequence Dictates the dG Targets of AFB₁-Epoxide Binding*

	Preferred targets Category I	Least Favored Targets Category III
5	*	*
	GGGG	AGAG
	GGGC	AGTG
10	GGGA	AGAA
	GGGT	AGAC
		AGAT
	CGGG	TGAG
	AGGG	TGAC
15	TGGG	TGAA
		TGAC
	CGGC	TGTG
	AGGC	TGTA
20	TGGC	TGTC
		TGTT
	CGGA	
	AGGA	
	TGGA	
25	CGGT	
	AGGT	
	TGGT	

30 *This table represents the dG targets of AFB₁-epoxide binding observed in studies with linearized double stranded PM-1 DNA. Moderately preferred dG targets, i.e. Category II, are omitted here but are described elsewhere (Modali and Yang, 1986).

Table 3. Predicted dG Targets within the Nucleotide Sequence of hhc^N Preferentially Attacked by AFB₁-Epoxide

[illegible]

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Table 3. (con't)

	*	*	*	*	*	*	*	*	*	*	
	CGCC	CGGC	GGCC	GGGC	GGGA	AGGA	TGCC	TGCG	TGGA	TGGG	GGAC
5		*			*	*			*	*	
		CGGC			GGGA	AGGA			TGGA	TGGG	
10			2205		2064	2094					
									2315		
									2331		
15				2352							
				2352							
			2482						2460		
									2718		
20							2797				
								2884			
							2926				

In order to analyze the possible effect of any such AFB₁ induced dG-->T mutation, site-targeted mutagenesis study of the hhc^M DNA was initiated using polynucleotides of 20 mers that carried a predicted dG-->dT point-mutation, presumably the result of an AFB₁-epoxide mutagenesis. Thus far, only a few of the predicted dG-->dT mutagenesis sites have been analyzed and these are summarized in Table 4. The recombinant construct carrying the hhc^M sequence in the SV40 T antigen vector plus a neomycin resistance marker, rpN^rPM-1 was used in this study since it offered the advantage of selecting the transfected cells by its resistance to Gentamicin sulfate (G418), an analog of neomycin. Using expression of hhc^M specific mRNA as a criterion, we analyzed by Northern dot-blot in a semi-quantitative assay of the mRNA, i.e. poly A enriched RNA, expressed in the G418 resistant NIH/3T3 cells after transfection with the mutagenized hhc^M sequence. Focal transformation in these cells was monitored for 4 to 6 weeks.

Results from seven mutagenized clones, for which nucleotide sequence confirmation was available, suggested that, thus far, mutation leading to a structural protein alteration did not seem to potentiate the cell-transformation of hhc^M (Table 4). Alternatively the introduced dG-->T mutations which led to amino acid substitution, thus far, have not altered cell-transformation or expression of mRNA levels. These included mutation at 577 which caused an amino acid substitution of Gly--->Val, and mutation at 1005 which resulted in no amino acid substitution because of the wobbling code.

Within the hhc^M nucleotide sequence, there exists an apparent open reading frame, ORF, coding for a polypeptide of about 467 amino acids. This was in good agreement with a 55-57 kD protein and some smaller polypeptide including one 53 kD protein observed in cell-free protein synthesis using hhc^M-specific mRNA in a rabbit reticulo-

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cyte lysate system. dG--->T mutations at nucleotide 73 and 74 in the 5' terminus, which bears the consensus sequence for ribosomal RNA binding site just 5' ahead of the first methionine codon, blocked cell transformation although hhc^M specific mRNA level showed no difference. This could be the result of blocking protein synthesis. Likewise, interpreted as a mutations at 492 and 550 also blocked cell-transformation since a stop codon (UGA) was introduced in each case to stop protein synthesis prematurely.

It was of interest to note that dG-->T mutation at 626 generated a sequence resembling the enhancer sequence for RNA polymerase II, which was reported to function even within the coding sequence (footnote of Table 4). The level of mRNA level was increased by 1.5 fold and cell transformation seemed to be enhanced by a slight increase in the number of foci per µg of DNA. This observation suggested that one possible action by which AFB₁ induced mutation in hhc^M, which itself is a moderately transforming DNA sequence, led to increase in its transformation potential is through augmentation of hhc^M expression. This is analogous to other observations which also indicated that an elevated expression of the cellular ras proto-oncogene driven by a murine LTR sequence, containing both promoter and enhancer sequence, also led to cell transformation in tissue culture cells predisposed to immortality.

Table 4. The Effect of dG --> dT Mutation Induced by Site-Targeted Mutagenesis Within The hhc^M DNA Sequence

5	# on hhc ^M	Sequence	mRNA Synthesis [#]	Cell Transformation [#]
		*		
	73	AGGA --> ATGA	+	_@1
		*		
10	74	AGGA --> AGTG	+	_@1
		*		
	492	TGGA --> TGTG	+	-@2
		*		
	550	GGAG --> GTAG	+	-@2
15		*		
	577	GGGC --> GTGC	+	+
		*		
	626	GGGG --> GTGG	++	++@3
		*		
20	1005	TGCA --> TTCA	+	+
@1 Disruption of ribosomal RNA (16S) binding site: AGGA.				
@2 Creation of stop codon: UGA.				
@3 Creation of an enhancer sequence: GGTGTGGTAAAG				
(Watson et al., 1987; Dynan and Tjian, 1985; Schaffner et al. 1985) and hence increases expression.				
# Cell transformation was determined by transfection analysis as described in Methods and mRNA synthesis in transfected cells was determined by Northern dot-blot analysis with [³² P]3.1 kb hhc ^M DNA.				

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Example III

Hhc^M-p52 and anti-p52 and their use as screening and diagnostic reagents for human hepatocellular carcinoma and related liver preneoplastic pathological conditions

5 Hhc^M-p52 as a fusion protein was produced by a bacterial system described above at high levels (Figure 5). This protein was used to generate a panel of both monoclonal and polyclonal antibodies against related human hepatoma proteins (see Figures 6A and 6B). Anti-p52, a
10 polyvalent antibody against hhc^M-p52 was produced and shown to be highly specific against an African (Mahlavu) hepatoma and a Philadelphia hepatoma (Figures 6A and 6B).

 Assays for the presence of hepatoma specific
15 protein p52 in tumor samples entail diffusion and immuno-precipitation using the tumor sample extracts reacted with anti-p52, with or without radioactive or immunofluorescence labels. Further, anti-p52, labelled with either a radioactive compound or with a chromophore, is useful in
20 RIPA or colorchange assays, respectively, for testing for the presence of hepatoma related proteins shed by the patient in sera and urine samples. Fluorescence imagery analysis using anti-p52 conjugated to a fluorescence compound or another suitable compound for systemic perfu-
25 sion, provide the ability to localize in situ preneoplastic or neoplastic lesions by scanning. Localization of lesions permits laser removal with surgical precision, and/or other treatment.

 Hhc^M-p52 nucleotide sequence, labelled appropri-
30 ately, can be applied to diagnose hepatomas in biopsy samples. Hhc^M-related nucleic acid sequences can be detected in needle biopsy samples of patients suspected of carrying preneoplastic nodules or liver cancer. This is accomplished by the using the polymerase chain reaction to
35 amplify "hhc^M-like" sequences using fragments of the hhc^M-p52 sequence as primers, and then detecting the presence of such hhc^M-like sequences in the biopsy sample with labelled hhc^M-p52 as a probe in a DNA-DNA hybridization

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reaction. Such an example is shown in Figure 7.

* * * *

The entire contents of all references cited herein are hereby incorporated by reference.

5 The present invention has been described in some detail for purposes of clarity and understanding. One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.
10

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WHAT IS CLAIMED IS:

1. A DNA fragment coding for the amino acid sequence set forth in Figure 1 or an allelic variation of said sequence, or a unique portion thereof.
- 5 2. The DNA fragment according to claim 1 wherein said fragment codes for the amino acid sequence set forth in Figure 1, or a unique portion thereof.
3. A recombinant DNA molecule comprising:
 - i) a vector, and
 - 10 ii) said DNA fragment according to claim 1.
4. The recombinant molecule according to claim 3 wherein said DNA fragment codes for the amino acid sequence set forth in Figure 1, or a unique portion thereof.
5. The recombinant DNA molecule according to
15 claim 3 further comprising a promoter sequence operably linked to said DNA fragment.
6. A host cell transformed with the recombinant DNA molecule according to claim 5.
7. The host cell according to claim 6, wherein
20 said cell is a procaryotic cell.
8. The host cell according to claim 7, wherein said cell is an E. coli cell.
9. A nucleotide fragment sufficiently complementary to said DNA fragment according to claim 1 to hybridize therewith.
25
10. A protein having the amino acid sequence set forth in Figure 1 or an allelic variation of said sequence, or a unique portion thereof.
11. The protein according to claim 10 wherein
30 said protein has the amino acid sequence set forth in Figure 1, or a unique portion thereof.
12. Antibodies specific for said protein according to claim 10.
13. The antibodies according to claim 12, wherein
35 said antibodies are polyclonal.
14. A process of producing the protein according to claim 10 comprising
 - culturing a host cell transformed with a

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recombinant DNA molecule comprising:

- i) a vector, and
 - ii) a DNA fragment coding for said protein under conditions such that said DNA fragment is expressed and said protein thereby produced; and
- isolating said protein.

15. A method of detecting the presence of the protein according to claim 10 in a sample comprising:

- i) contacting the sample with an antibody specific for said protein under conditions such that binding of said antibody to said protein can occur, whereby a complex is formed; and
- ii) assaying for the presence of said complex.

16. The method according the claim 15 wherein said antibody is linked to a detectable label.

17. A method of detecting the presence of a nucleotide sequence coding for said protein according to claim 10 in a sample comprising:

- i) contacting the sample with a nucleotide fragment sufficiently complementary to said nucleotide sequence to hybridize therewith under conditions such that hybridization can occur, whereby a complex is formed, and
- ii) assaying for the presence of said complex.

18. The method according to claim 17 wherein said nucleotide fragment is linked to a detectable label.

19. A method of diagnosing the presence of hepatocellular carcinoma in a patient comprising:

- i) contacting a biological sample from said patient with an antibody according to claim 12 under conditions such that binding of said antibody to said protein present in said sample can occur, whereby a complex is formed; and
- ii) assaying for the presence of said complex.

20. The method according to claim 19 wherein said sample is a tissue sample.

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21. A method of diagnosing the presence of hepatocellular carcinoma in a patient comprising:

5 i) contacting nucleic acid sequences derived from a cellular sample from said patient with said nucleotide fragment according to claim 9 under conditions such that hybridization can occur, whereby a complex is formed; and

ii) assaying for the presence of said complex.

10 22. A diagnostic kit for detecting the presence of the protein according to claim 10 in a sample comprising a container means having disposed therewithin antibodies specific for said protein.

15 23. A diagnostic kit for detecting the presence of a nucleic acid sequence coding for a protein having the amino acid sequence set forth in Figure 1 or an allelic variation of said sequence, or a unique portion thereof, comprising a container means having disposed therewithin said nucleotide fragment according to claim 9.

27	AAG CTT AAT AGA AAA TAT GAG CAA CAT ACA CAA ACA TTA GCA ACA ATG ATA TAA AAT ACC ACT TAA ACA TAA GGA AAA ATG	81
		MET
108	TTG CCC TTC ACT TGT GGA AGA AAT GCA AAT GAA AAC AGC CCT AGG GAT GTT GAC GGT GGG GTG GCA CCT GCT GCA GAG GGT	162
	Leu Pro Phe Thr Cys Gly Arg Asn Ala Asn Glu Asn Ser Pro Arg Asp Val Asp Val Gly Val Ala Pro Ala Ala Glu Gly	
189	AAC GTG CAG CAT GTC GAG GGC AGC ACT GCC AAG GCT GGT TTG AGC TCA AGG TCA GGT GGA GGT AGT CTC TCC CAT CTC	243
	Asn Val Gln His Val Glu Gly Ser Thr Ala Lys Ala Gly Leu Ser Ser Arg Ser Gly Gly Gly Ser Leu Ser His Leu	
270	TTC TGC GAG TGC AGC TCT AAA CCC TGC CTG AAA CAC CAC GTG GAG AAG CTA TCT GAG CTG CCT CCA GGA CAC ATG CAA ATG GAC	324
	Phe Cys Glu Cys Ser Lys Pro Cys Ser Lys Lys His Val Glu Lys Leu Ser Glu Leu Pro Pro Gly His MET Gln MET Asp	
351	ACT CTG ATC ATA AAA TTA TCA GGA AGA TTG AGA AAT AAG ACA AAA ATG GAG GTG CCA CCA AAC CAG TGG AAA TTT TTC CCC	405
	Thr Leu Ile Ile Lys Leu Ser Gly Arg Leu Arg Asn Lys Thr Lys MET Glu Val Pro Pro Asn Gln Trp Lys Phe Phe Pro	
432	TTT TCA TTC CTC TGG CAT TCC CTG GCC TTG ACT CAA GGC AGC CCA CAC TCT AGG AGC AGA CAC CAG GGC ACA GGT GGG GAG	486
	Phe Ser Phe Leu Trp His Ser Leu Ala Leu Thr Gln Gly Ser Pro His Ser Arg Ser Arg His Gln Gly Thr Gly Gly Glu	
513	CTC TGG GGG ACC CTC CAG GCT TAC TCA GTG AAT GGG TTA GCA GCA GCC ACA GGA GCC ACC ATG GAG CCT GCA GGG ACC CAC	567
	Leu Trp Gly Thr Leu Gln Ala Tyr Ser Val Asn Gly Leu Ala Ala Thr Gly Ala Thr MET Glu Pro Ala Gly Thr His	

FIG. 1

594 AAC ACT GAG GGC AGG GAT CTT GCC TCT AAT CAG ATA AGC TGT GAT TCC CGA GAG GGT GGG GTA AAG GCC ACG GGT CTT TTT 648
 Asn Thr Glu Gly Arg Asp Leu Ala Ser Asn Gln Ile Ser Cys Asp Ser Arg Glu Gly Gly Val Lys Ala Thr Gly Leu Phe
 675 CTC TCC ACA TCT TCC CAC GTC ATG ACC CCA GAG GGT CGA AGA GGG AGA AAG TGT GAG CAC CGT GAC ATA ATG AGC CGC AGC 729
 Leu Ser Thr Ser Ser His Val MET Thr Pro Glu Gly Arg Arg Gly Lys Cys Glu His Arg Asp Ile MET Ser Arg Ser
 756 CTT CTG ACT AGA TGC CCC AAA GAA GAA TCC CAG GTG ACC ACA CAG CAT CAG AGA AAC TGC AGG GTA ATG AGG AAC TTT GGA 810
 Leu Leu Thr Arg Cys Pro Lys Glu Glu Ser Gln Val Thr Thr Gln His Gln Arg Asn Cys Arg Val MET Arg Asn Phe Gly
 837 AAG CAA TCC ATC GTG TTG TCA GTA AAA CCT CTG GCT CAC TCC CGA GCT GGG CAT GCA TGG ATG GTG ACC CTC GAT GGA ATA 891 2/11
 Lys Gln Ser Ile Val Leu Ser Val Lys Pro Leu Ala His Ser Arg Ala Gly His Ala Trp MET Val Thr Leu Asp Gly Ile
 918 GAC TAT GAG GAA CCA GGT GAG GGG ATC TAC CTC CAC CGA GAC GTG AGA GTG ACC TGC ATA CCC AAA CAC CAT GAG GCT TTA 974
 Asp Tyr Glu Glu Pro Gly Glu Gly Ile Tyr Leu His Arg Asp Val Arg Val Thr Cys Ile Pro Lys His His Glu Ala Leu
 999 AAG ACT GAG CTG ATG TGG AAG CCA CAG CCT CTG CAG GTT GCT CTG CAC TTG CAA CAT AAG CCC AAC CAC ATC AAT TGC TGC 1053
 Lys Thr Glu Leu MET Trp Lys Pro Gln Pro Leu Gln Val Ala Leu His Leu Gln His Lys Pro Asn His Ile Asn Cys Cys
 1080 AAA ACA AAA CTA CAG CAT TCT CCA TAC CAC TTA AAT AAG ACA CAG AGT CTC ACA ACA TTC AAA ACG CCC AGG ACA CAA TCC 1134
 Lys Thr Lys Leu Gln His Ser Pro Tyr His Leu Asn Lys Thr Gln Ser Leu Thr Thr Phe Lys Thr Pro Arg Thr Gln Ser

FIG. 1(cont.)

1161 AAA ATT ACT TCT ACA AAA AAT CAG GAA AAT CTC AAT GAG CAA GGA AAA TGG CAA TCA GTA GCT GCC AGT GCT GAG ATG ACA
 Lys Ile Thr Ser Thr Lys Asn Gln Glu Asn Leu Asn Glu Gln Gly Lys Trp Gln Ser Val Ala Ala Ser Ala Glu MET Thr 1215
 1188
 1248 ATG AGG GTT GGA ATC ATC AAC ATC TTT AAA GTA ATT ATC ATA AGC ATT CTC CAG CAA GTA ATG GCA AAC ACT CTT GAG ATA
 MET Arg Val Gly Ile Ile Asn Ile Ile Asn Val Ile Ile Ser Ile Leu Glu Gln Val MET Ala Asn Thr Leu Glu Ile 1296
 1270
 1323 AAT GGA AAG ATA AGA AGG CTC AGG GAG AAA GTG GAA TGT ACA AAG AAT GAC CAA GTG GGA ATT GCA CCA CTG GAA ACA AAT
 Asn Gly Lys Ile Arg Arg Leu Arg Glu Lys Val Glu Cys Thr Lys Asn Asp Gln Val Gly Ile Ala Pro Leu Glu Thr Asn 1377
 1350
 1404 CAC CAG GAT AAA GCA GTC TCT GGC TGG GCC AAC AGG AGA ATG GAA ATG AAA AGG GAA AGA GTT GTT ATG GCA GTT GTC CAA
 His Gln Asp Lys Ala Val Ser Gly Trp Ala Asn Arg Arg MET Glu MET Lys Arg Glu Arg Val Val MET Ala Val Val Gln 1458
 1431
 1485 → TTT GAA CAA CAC AAA AGA CAC TGA TTT AAA AAA AAA TGA GGC AGG GCT CAG TGG CTC ACA CCG ATA ATC CCA ATA CCT TGG
 Phe Glu Gln His Lys Arg His 1539
 1512 *
 1566 GAG GCC GAG GCA ATG TAT CAC CTG AGG TCA GGA GTT CAA GAC TAC CCT GGC CAA CAT GGC AAA ATC CCA TCT CTA CTG AAA
 Tyr His Leu Arg Ser Gly Val Gln Asp Tyr Pro Gly Gln His Gly Lys Ile Pro Ser Leu Leu Lys 1620
 1647 ATA CAA GAA TTA GCT GGG CAT GGT GGC AGG TGC CTG CAA TCC CAG CTA CTC AGG AGG CTG AGG CAG GAG AAT CAC TTG AAC
 Ile Gln Glu Leu Ala Gly His Gly Gly Arg Cys Leu Gln Ser Gln Leu Leu Arg Arg Leu Arg Gln Glu Asn His Leu Asn 1701
 1674

FIG. 1(cont.)

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1728 TCG GGA GGT AGA GGG TGC AGT GAG CCA AAA TCG CAC CTC TGC ATT CCA GCC TGG GTG ACA GAG GGA GAC TCT GTC TCA AAA
 Ser Gly Gly Arg Cys Ser Glu Pro Lys Ser His Leu Cys Ile Pro Ala Trp Val Thr Glu Gly Asp Ser Val Ser Lys 1782
 1809 CAA AAC AAA ACA AAT GAA CAG CAC CTC AGG AAC AAT ACC AAA AAG TCC AAC AGC TGT ATA ATT GGT GGC CCA GAA GGA
 Gln Asn Lys Thr Lys Asn Glu Gln His Leu Arg Asn Asn Thr Lys Lys Ser Asn Ser Cys Ile Ile Gly Gly Pro Glu Gly 1863
 1890 GAG GAG AAA GAG TGG AGT ACA GAA ATG AGA TCT GAA GAA CTA ATG ACT GAT AAT GTT TCA ATT TTG AAA AAG GAC ATA AAC
 Glu Glu Lys Glu Trp Ser Thr Glu MET Arg Ser Glu Glu Leu MET Thr Asp Asn Val Ser Ile Leu Lys Lys Asp Ile Asn 1944
 1971 CTA AAG ATT ATA GAT TCA AAA GCC CAG CTG AAT TCA AAT AAG ATA AAT ACA GAT GCA GAT ATA TTA TCA TTA AAC TGT GAA
 Leu Lys Ile Ile Asp Ser Lys Ala Gln Leu Asn Ser Asn Arg Ile Asn Thr Asp Ala Asp Ile Leu Ser Leu Asn Cys Glu 2024
 2052 ATA AAT TGG TTT TGT CAC AAG CCA GCA TTG TCA CTG TGG GAG AAA AGA GAT CAA AAG TAC ACA AGG AAG GAA GGA AAT ACA
 Ile Asn Trp Phe Cys His Lys Pro Ala Leu Ser Leu Trp Glu Lys Arg Asp Gln Lys Tyr Thr Arg Lys Glu Gly Asn Thr 2106
 2133 GAA TAT TAT GGC CAT GGG AAA GAG GTG TCA GTG TGA ATA CAT AGA ACA GCA CAC TTA AGC AAC AAC CCC AAA TGA TGG GGC
 Glu Tyr Tyr Gly His Gly Lys Glu Val Ser Val • Ile His Arg Thr Ala His Leu Ser Asn Asn Pro Lys • Trp Gly 2187
 2214 TTC CTA CAA AAC AGT TGG CCT TTA CTC TTC AAA AGT GTC AGG TCA CGA AAT AAA TCC ATG CTG AGG ACC CGT TCC AGG TTA
 Phe Leu Gln Asn Ser Trp Pro Leu Leu Phe Lys Ser Val Arg Ser Arg Asn Lys Ser MET Leu Arg Thr Arg Ser Arg Leu 2268

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FIG. 1 (cont.)

2295 AAG CAG ACT AAA GGG GCT GGA CAA CAC AGT GAA ACG TGT GAG CTT GGA TTA GAT ATA TGC TGG ACT AGA GAA GGC TGT GAG
 Lys Gln Thr Lys Gly Ala Gly Gln Pro Ser Glu Thr Cys Glu Leu Gly Leu Asp 2322 2349
 2376 GGG GAC AAT GGC TGA AAT GTG AAT GAG GTT ACT ATA GTT ATG AAA AAT GTT AAG ACT TGG AAA ATC TAT ATA AAG CAG ACG
 2457 2484
 2511 GCA TAA TTC TTG TAC TTT TTT TGC AAC TTT TTA ATA AAC CTG AAA CTA TTT CAA AAT GAA AAG TTA ATC CAA GCT GTC TTG
 2538 2565
 2592 AGT AGA AGT TAA AAC AAC AAC AAA AGA AAA TTG AAA AGT TAA AAA TGA ACC CCC AAC AGA ATG TTC CCC TTT ATT TTT
 2619 2646
 2673 CTT TCA TGT AAG GAC GCA GGA TAT GCA TTT TGC TCA GCT ACC ACC CTT CAC TGC ATC CCA TTT TGA GAA GTG GTA TTT TCT
 2700 2727
 2754 TCA TTC ATC TGT TCT AGG TTT TTA AAA AAA TAT TTA AGA TCT TCT CTT TTT AAA GAA TCT GTT CAT TTG GAA TGT ACT TTT
 2781 2808
 2835 TGC ATT TTT ACT TGT GAA AAT ATG TAT TTA TCC TTT TTG TTA TGA ATG TAT GAC TTC ACT GTG TCA GAG AAT ATG GTC TTA
 2862 2889
 2916 AGA GAT ACA GAA AAC TTT TGA GAA TGA TAA GAT CTG GAC ATG CTA GAT GAA ATC AAA GCC CTG GAT GTC CTT GTT CAA GCT T
 2943

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FIG. 1 (cont.)

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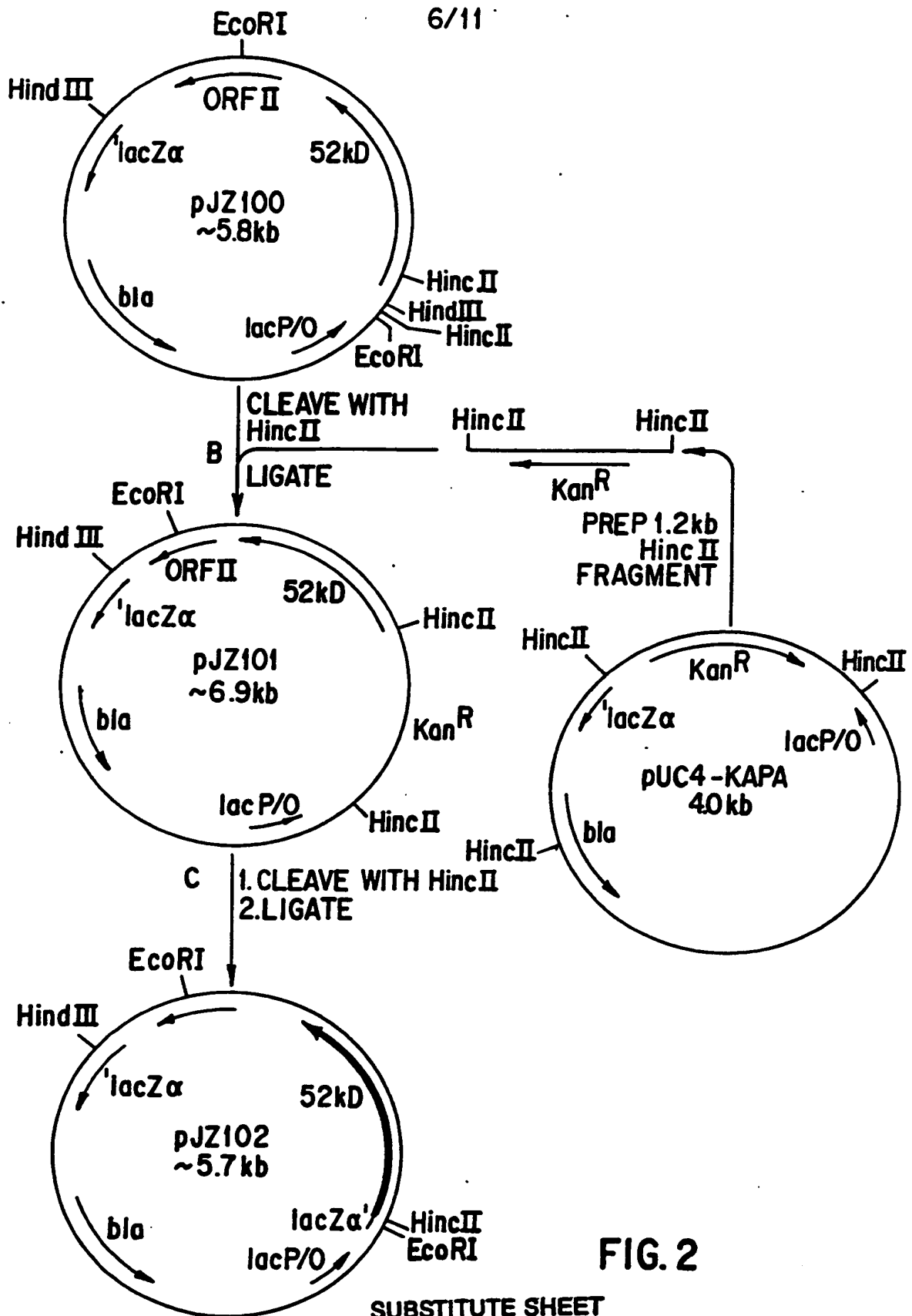


FIG. 2

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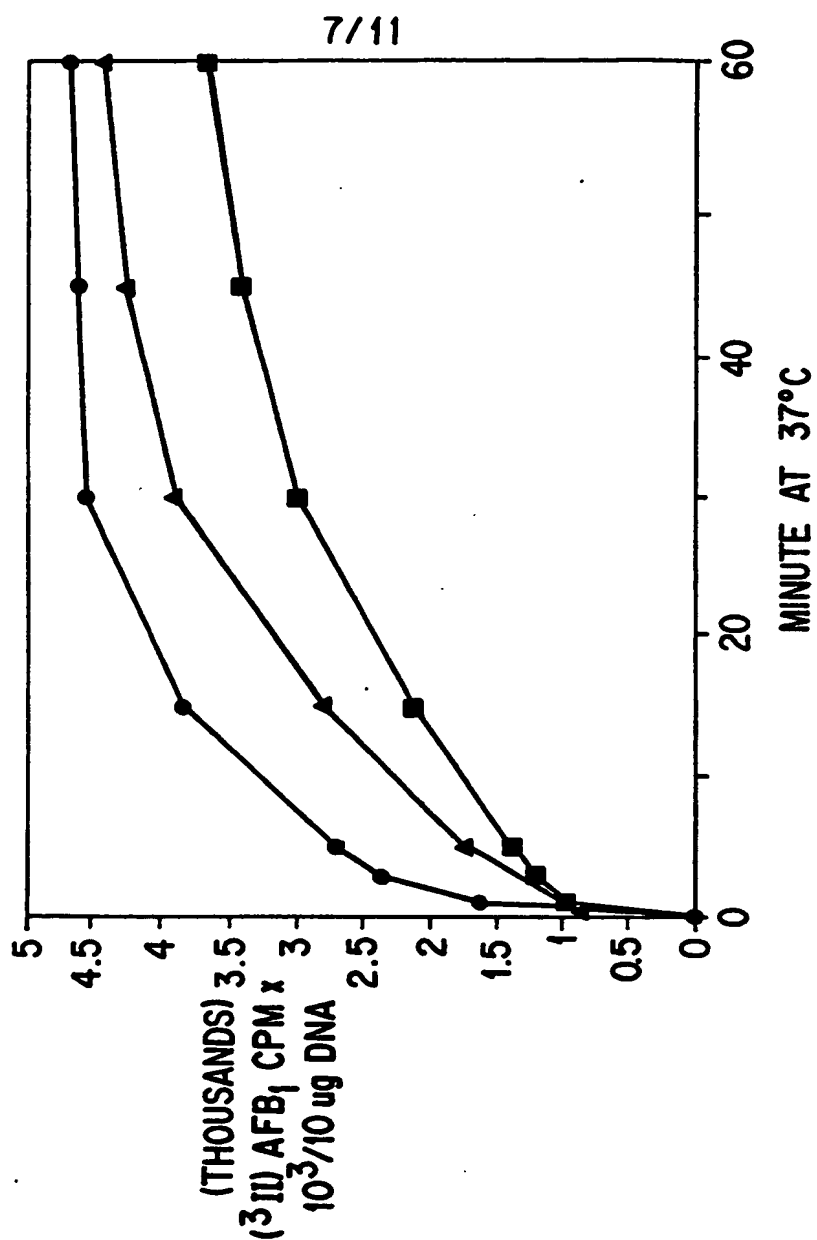


FIG. 3

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FIG. 4



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m g g f f e e d d c m b a

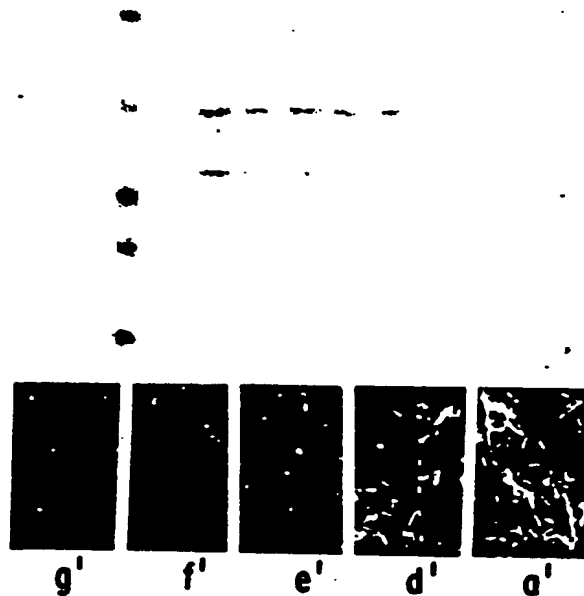


FIG. 5

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FIG. 6B

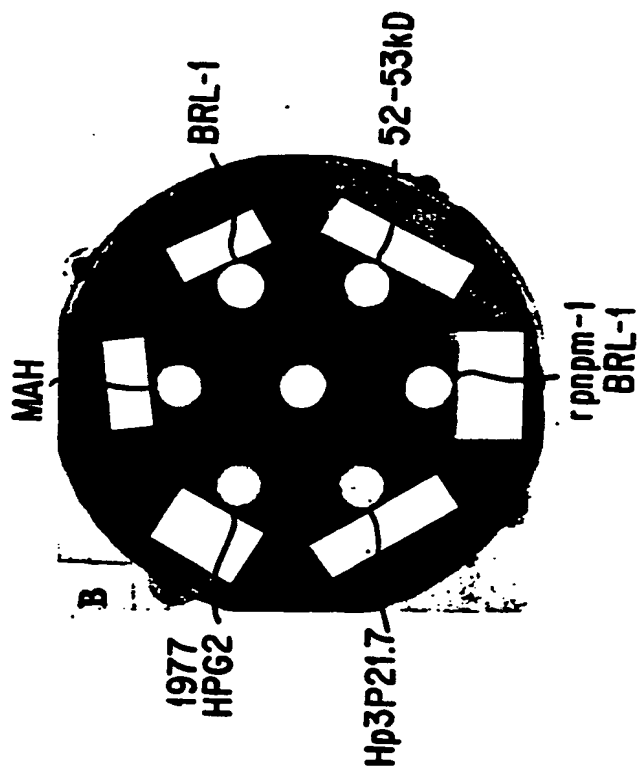
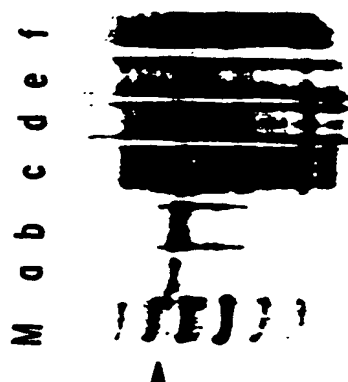


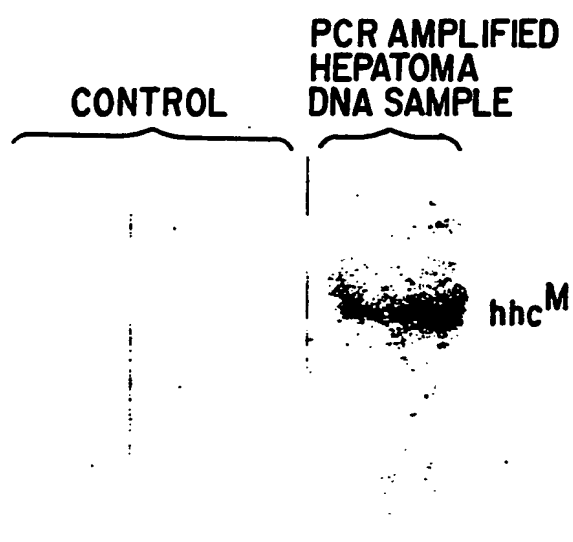
FIG. 6A



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FIG. 7



**DNA-DNA HYBRIDIZATION
AGAINST (³²P)-hhc^M DNA**

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US90/07333**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C07H 19/12; C12Q 1/68; C12N 15/00 U.S. Cl. 536/27; 435/6; 935/77, 78								
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border: 1px solid black; padding: 5px;">Classification System</td> <td style="border: 1px solid black; padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">U.S. Cl.</td> <td style="border: 1px solid black; padding: 5px;">536/27; 435/6; 935/77, 78</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸</div> <p style="margin-top: 10px;">APS, STN, Gen Bank, EMBL</p>			Classification System	Classification Symbols	U.S. Cl.	536/27; 435/6; 935/77, 78		
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border: 1px solid black; padding: 5px;">Category ¹⁰</th> <th style="width: 70%; border: 1px solid black; padding: 5px;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border: 1px solid black; padding: 5px;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="border: 1px solid black; padding: 10px; vertical-align: top;">A</td> <td style="border: 1px solid black; padding: 10px;">Journal of General Virology, Volume 63, issued 1982, Yang et al, "Molecular Cloning of the Endogenous Rat C-type Helper Virus DNA Sequence: Structural Organization and Functional Analysis of Same Restricted DNA fragments" pages 25-36. <i>Some</i></td> <td style="border: 1px solid black; padding: 10px; vertical-align: top;">1-23</td> </tr> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	A	Journal of General Virology, Volume 63, issued 1982, Yang et al, "Molecular Cloning of the Endogenous Rat C-type Helper Virus DNA Sequence: Structural Organization and Functional Analysis of Same Restricted DNA fragments" pages 25-36. <i>Some</i>	1-23
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"d" document member of the same patent family</p> </div> </div>								
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">28 January 1991</div> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold; font-size: 1.2em;">20 FEB 1991</div> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;"> International Searching Authority <div style="text-align: center; font-weight: bold;">ISA/US</div> </td> <td style="border: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;"> <i>Mindy B. Fleisher</i> Mindy B. Fleisher </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">28 January 1991</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold; font-size: 1.2em;">20 FEB 1991</div>	International Searching Authority <div style="text-align: center; font-weight: bold;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;"> <i>Mindy B. Fleisher</i> Mindy B. Fleisher </div>		
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